

The University of Chicago

THE EFFECT OF PENICILLIN ON GROWTH
AND TOXIN PRODUCTION BY ENTERO-
TOXIC STAPHYLOCOCCI

A DISSERTATION SUBMITTED TO THE FACULTY OF THE
DIVISION OF BIOLOGICAL SCIENCES IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

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MILTON SEGALOVE

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INTRODUCTION

The effect of antibiotics on staphylococci and other microorganisms has been the subject of a large number of investigations. Many reports in the literature have indicated the susceptibility of staphylococci to penicillin (Rammelkamp and Maxon, 1942; Bondi and Dietz, 1944; Spink, Ferris and Vivino, 1944; Rutherford and Crowson, 1945; Bondi and Dietz, 1945; Demerec, 1945; Mason, 1945; Blair, Carr and Buchman, 1946, and others) as well as the resistance of some strains (Demerec, 1945; Kirby, 1944; Gots, 1945; Bondi and Dietz, 1944a) which has been attributed by Demerec (1945), to selection of resistant cells and by others to production of penicillinase. The mechanisms of resistance of these and other microorganisms to the antibiotic have also been reported (Bigger, 1944; Kirby, 1944; Bondi and Dietz, 1944a; Demerec, 1945; Gots, 1945; Bondi and Dietz, 1945; Spink and Ferris, 1945; Woodruff and Foster, 1945; Luria, 1946; Klein and Kimmelman, 1946).

However, few studies have appeared concerning the action of penicillin on bacterial toxins. Neter (1945) reported no effect of the antibiotic on tetanus toxin; Ercoli, Lewis and Moench (1945)

obtained no neutralization of diphtheria toxin *in vivo* but did find that high concentrations of penicillin were able to neutralize the toxin *in vitro*; and Blair, Carr and Buchman (1946) found that penicillin neither inhibited the formation nor was able to destroy staphylococcus alpha-hemolysin. Some of the first positive results were obtained by Boor and Miller (1945) and Miller and Boor (1946) who succeeded in protecting laboratory animals against gonococcal and meningococcal endotoxins by injecting large amounts of penicillin into the infected animals.

Although the role of the staphylococcus as the most common cause of food poisoning has been well established (Dack, Cary, Woolpert and Wiggers, 1930; Jordan, 1930; Dolman, 1934) and the enterotoxic effects have been shown to be attributable to the production of a toxic substance, the nature of the enterotoxin has remained obscure and methods for the control of staphylococcal food poisoning are incomplete. Furthermore, no satisfactory method of differentiation of food poisoning staphylococci other than by the demonstration of enterotoxin is known. The ubiquitous distribution of the staphylococcus and the difficulties in identification of food poisoning strains have largely limited existent control measures to application of adequate methods of refrigeration, and less generally, to supervision of food handlers.

The susceptibility of staphylococci to penicillin and the effects of penicillin on bacterial toxins demonstrated by others suggested the desirability of an investi-

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gation of the effects of this antibiotic on food poisoning staphylococci and their toxins. It seemed possible that such an investigation would be of interest both from the point of view of the control of staphylococcal food poisoning either through preventing growth of the organisms in contaminated food or by neutralization or destruction of toxicity, or, as suggested by the reports of Mason (1945) and Rutherford and Crowson (1945), that penicillin resistance might offer a means of segregation of food poisoning strains of staphylococci.

Consequently, an investigation has been made of (1) the natural susceptibility of enterotoxic strains of staphylococci to penicillin, (2) the development of artificial resistance of such strains to penicillin, (3) the determination of the effect of penicillin on toxin formation by naturally and artificially resistant strains and (4) the effect of the antibiotic on certain biochemical activities of staphylococci.

The resistance of staphylococci to penicillin

The natural resistance of staphylococcus strains to penicillin—The natural resistance to penicillin of fifteen enterotoxic and five non-enterotoxic laboratory strains of staphylococci has been determined. Prior to this investigation, these cultures had been maintained on veal infusion agar slants and none had had previous contact with penicillin. Furthermore, in the course of this study the bacteria were in contact with the antibiotic only during the actual tests and were otherwise stored on penicillin-free mediums.

Materials and Methods.—The strains of staphylococci tested for natural resistance to penicillin were selected from the culture collection maintained in this laboratory. The enterotoxic strains were known to be enterotoxigenic by previous tests, whereas the non-enterotoxic

strains have given consistently negative results in similar tests for enterotoxin (Fig. 1). Strains of *Staphylococcus aureus* no's. 43, 147, 161 and 196, which were used in the majority of the experiments, have the following histories:

No. 43, well known as the Wood strain no. 86. It has been negative for enterotoxin when fed to man, monkeys and kittens in tests conducted over many years.

No. 147, was received from Dr. William Litterer, State Department of Health, Nashville, Tennessee on November 14, 1933. It was isolated from a food poisoning outbreak and has been enterotoxic by monkey feeding and intravenous cat injection tests in this laboratory.

No. 161, was isolated by Dack, Bowman and Harger (1935) from tongue sandwiches causing a food poisoning outbreak. It produces potent enterotoxin.

No. 196, was received from Dr. G. G. Slocum in August, 1940 and was isolated from ham involved in food poisoning. Kitten tests at time of isolation and monkey feeding tests conducted in this laboratory have all been positive for enterotoxin.

Tests for sensitivity to penicillin were performed by the tube dilution method adapted from that of Kolmer (1945). The tests were conducted in Amigen* liquid medium made according to the following formula: pancreatic hydrolysate of casein (Amigen)—10.0 gms, thiamine hydrochloride—0.05 mgs, nicotinic acid—1.20 mgs, calcium pantothenate—1.0 mg, MgSO_4 —14.0 mgs, KH_2PO_4 —4.536 gms, $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ —20.0 mgs, glucose—2.50 gms, 4 per cent NaOH—35.0 ml, and distilled water to make 1,000 mls. The p_{H} was adjusted to 7.6 with NaOH and the medium was sterilized in the autoclave.

The penicillin used in all experiments was commercial penicillin sodium obtained from two firms† and was packed in ampoules containing 100,000 units each. The antibiotic was dissolved either in phosphate buffer, p_{H} 7.2, or in Amigen liquid medium usually just before use and residual solutions were not used if stored for longer than one week in the refrigerator. No decrease in penicillin potency was observed during this storage period. From the initial solution containing 10,000 units per ml of penicillin, dilutions containing lesser concentrations were prepared aseptically for the experiments.

Tubes containing 2-fold dilutions of penicillin

* Pancreatic hydrolysate of casein, vitamin- and carbohydrate-free. Product of Mead Johnson and Co.

† E. R. Squibb and Sons, New York, and Lederle Laboratories, New York.

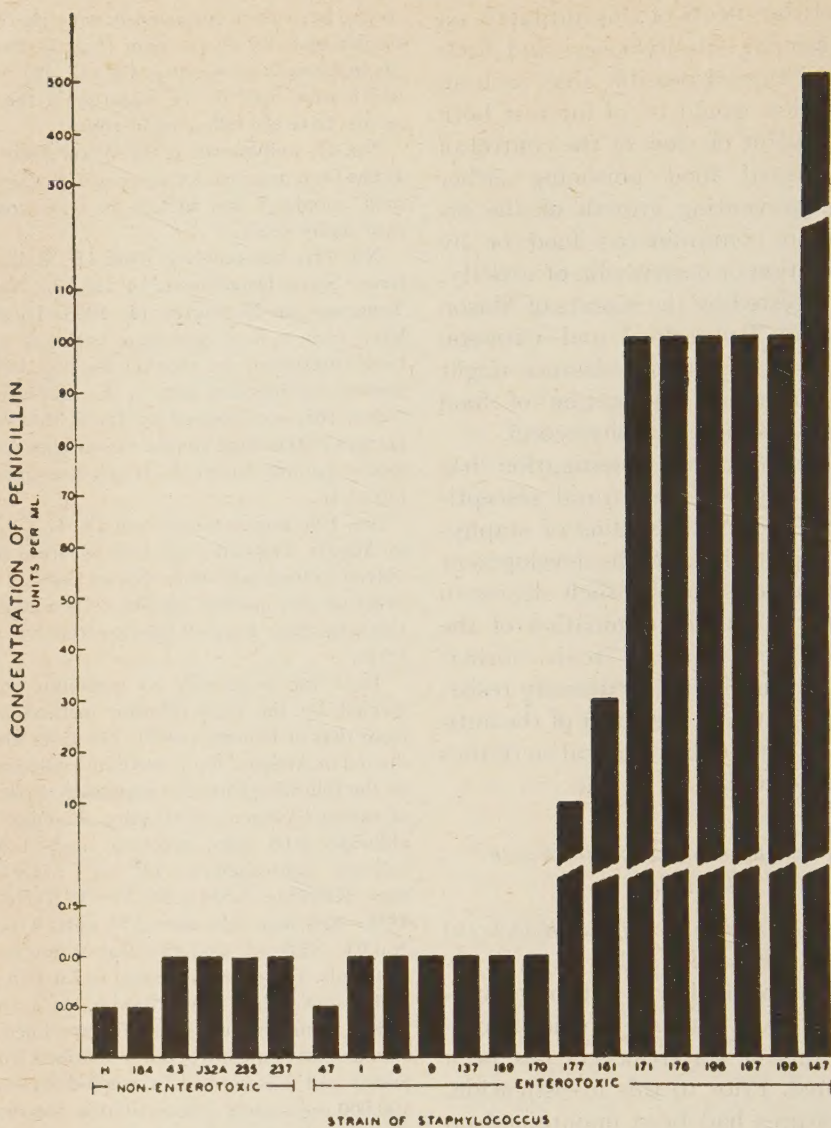


FIG. 1.—Natural resistance of staphylococcus strains to penicillin.

were inoculated with 0.1 ml of a 1:100 dilution of a 24 hour Amigen liquid culture of the strain to be tested and the final volume brought to 1.0 ml with the same medium. Growth was determined by turbidity of the cultures after incubation for 24 and 48 hours at 37 C. The sensitivity of the strains was compared with that of the standard, *Staphylococcus aureus*, Oxford strain H (Florey and Jennings, 1942). Repeated tests were done with all cultures.

Results.—The results of these experi-

ments, shown graphically in figure 1, indicate that a much higher proportion of enterotoxigenic than non-enterotoxigenic strains was naturally resistant to penicillin. Few authors have reported such high resistance in strains of staphylococci previously unexposed to penicillin. Rutherford and Crowson (1945) reported a strain of *Staph. aureus* (isolated from ham implicated in an outbreak of

food poisoning) which was resistant to 12.5 units per ml. and which produced a smooth variant resistant to 100 units per ml. of penicillin. Mason (1945) also reported three food poisoning strains of staphylococci resistant to 650 units per ml. On the other hand, Rammelkamp and Maxon (1942) reported that 25 of 29 strains of staphylococci studied were killed by concentrations of 0.04 to 0.08 units per ml of penicillin, and Spink, Ferris and Vivino (1944) studied 68 strains of coagulase-positive staphylococci and found that, of this number, 8 strains were comparatively resistant requiring 0.4 to 0.8 units per ml of penicillin for inhibition of growth. Recently, Blair, Carr and Buchman (1946) tested 79 strains of staphylococci and found that 62 (78.5%) were sensitive to 0.2 units or less per ml and 17 (21.5%) were inhibited by concentrations ranging from 0.3 to 50.0 units per ml of penicillin. Of these latter 17 strains, two were resistant to 20 and two were resistant to 50 units per ml.

It is evident that the natural resistance of staphylococci to penicillin is highly variable and food poisoning strains as a group cannot be distinguished by their penicillin resistance, since in the present experiments strains were encountered sensitive to 0.05 and 0.1 units per ml.

The development of artificial resistance to penicillin.—Many authors have reported the development of artificial resistance to penicillin by staphylococci both in vivo and in vitro (Demerec, 1945; Gallardo, 1945; McKee and Houck, 1943; North and Christie, 1945; Rake, McKee, Hamre and Houck, 1944; Rammelkamp and Maxon, 1942; Spink and Ferris, 1945, Spink, Ferris and Vivino, 1944; Todd, Turner and Drew, 1945). In the present studies, two strains of staphylococci, one enterotoxic (no. 161) and one non-enterotoxic (no. 43),

were selected for experiments on the development of increased resistance to penicillin.

In the initial experiments, an attempt to increase resistance was made by growing the stock strain on veal infusion agar containing varying concentrations of antibiotic. This method was relatively unsuccessful and highly resistant derivatives were obtained only by successive transfers in Amigen liquid medium containing progressively increasing concentrations of penicillin. A final resistance of strains no. 161 and 43 to 2,000 and 10 units per ml respectively was obtained, representing an increase of 66 and 100 times the resistance of the parent cultures. These artificially resistant strains hereinafter referred to as no. 161R and no. 43R and selected naturally resistant strains were used in further experiments. At various intervals of time, repeated tests were done on the stock cultures of the resistant strains to determine any loss of resistance to penicillin. No loss was found in strain no. 43R but strain no. 161R was found resistant to only 400 units per ml after 5 months storage in the refrigerator. Several passages in Amigen liquid medium containing penicillin brought about a rapid return to the former high resistance of 2,000 units per ml.

Mechanisms of resistance to penicillin.—Demerec's (1945) work on the development of resistance to penicillin in a strain of staphylococcus demonstrated that selection of resistant cells occurs through the action of the antibiotic as a selective agent. The experiments of Klein and Kimmelman (1946) on the development of streptomycin resistance in *Shigella* demonstrated a similar mechanism. Many other reports have appeared describing the presence and/or absence of a penicillin-inactivating substance (penicillinase) in staphylococci (Abraham and Chain, 1940; Florey et al., 1941; Kirby, 1944;

Bondi and Dietz, 1944; Bondi and Dietz, 1944a; Spink and Ferris, 1945; Woodruff and Foster, 1945; Bondi and Dietz, 1945; Gots, 1945). Since

TABLE 1.—Resistance of strain 147 to penicillin.

Inoculum (1.0 ml of culture dilution)	Bacterial colony counts					
	Penicillin (units per ml)					
	(control) 0	100	200	400	500	1,000
10 ⁻¹	*	*	*	*	*	*
10 ⁻²	*	*	*	*	*	136
10 ⁻³	*	90	52	0	0	0
10 ⁻⁴	*	0	0	0	0	0
10 ⁻⁵	*	0	0	0	0	0
10 ⁻⁶	*	0	0	0	0	0
10 ⁻⁷	59	0	0	0	0	0

* Colonies too numerous to count.

TABLE 2.—Resistance of strain 161R to penicillin.

Inoculum (1.0 ml of culture dilution)	Bacterial colony counts						
	Penicillin (units per ml)						
	(control) 0	50	200	500	1,000	1,200	1,400
10 ⁻¹	*	*	*	*	*	*	*
10 ⁻²	*	*	*	0	0	0	0
10 ⁻³	*	0	0	0	0	0	0
10 ⁻⁴	*	0	0	0	0	0	0
10 ⁻⁵	*	0	0	0	0	0	0
10 ⁻⁶	764	0	0	0	0	0	0
10 ⁻⁷	75	0	0	0	0	0	0

* Colonies too numerous to count.

TABLE 3.—Resistance of strain 43R to penicillin.

Inoculum (1.0 ml of culture dilution)	Bacterial colony counts						
	Penicillin (units per ml)						
	(control) 0	0.5	1.0	2.0	5.0	10.0	
10 ⁻¹	*	*	*	*	*	*	*
10 ⁻²	*	*	*	*	*	*	*
10 ⁻³	*	*	*	*	*	*	*
10 ⁻⁴	*	*	*	*	*	*	*
10 ⁻⁵	*	*	*	*	*	*	*
10 ⁻⁶	605	649	540	435	302	78	
10 ⁻⁷	57	64	46	43	38	12	

* Colonies too numerous to count.

the occurrence of selection and the ability to produce penicillinase both seem to have a role in resistance of staphylococci to penicillin, the following experiments were done to elucidate the mechanisms of resistance in the strains under investigation.

Materials and Methods.—The resistance of the bacterial population of the strains to be tested was determined by survival in agar pour plates containing penicillin. The bacterial suspension (1.0 ml) was added to the desired concentration of penicillin (1.0 ml volume) and immediately followed by 8.0 ml of melted agar cooled to 45 C. After 72 hours incubation at 37 C the colonies were counted. The growth of strains no. 43, 43R, 147, 196, 161 and 161R was tested in duplicate plates, by the above method, and experiments with strains no. 43R and 161R were repeated. The tabulated results are averages of counts obtained on duplicate plates.

Results.—The results of these experiments, shown in tabular form in tables 1, 2, and 3, indicate that neither strain no. 147 nor no. 161R was completely resistant to penicillin since no growth of bacteria occurred in high concentrations of the antibiotic when the number of bacteria in the inoculum was progressively reduced. The results obtained with strain no. 43R, on the other hand, seem to indicate a relative homogeneity in resistance to antibiotic, since the counts in plates containing penicillin were comparable to those in the control plates even when the inoculum contained few bacteria. Thus, these results with strain no. 43R are consistent with those obtained by Demerec (1945), indicating the selection of resistant cells by growth in penicillin-containing mediums. However, the results in experiments with strain no. 161R seemed to indicate another mechanism of resistance. An uncountable number of colonies grew on plates containing penicillin inoculated with large quantities of organisms, whereas the plates inoculated with the next higher ten-fold dilution showed no growth. The same type of result was obtained in similar tests with strain no. 147, a naturally resistant culture. In these latter experiments, the colony count seemed to indicate the production of a penicillin-inactivating substance which was pres-

ent in amounts sufficient to allow growth of non-resistant cells. The following experiments were done to determine the production of such a substance by the strains used.

The production of penicillinase.—Sterile filtrate of three-day cultures of strains to be tested (0.4 ml) was added to 2-fold dilutions of penicillin (0.5 ml volume) and the tubes were inoculated with 0.1 ml of a 1:100 dilution of a 24 hour Amigen liquid culture of *Staphylococcus aureus*, strain H. Control tubes were prepared by replacing the sterile culture filtrate with sterile Amigen liquid medium. All tests were incubated at 37 C for 48 hours.

Results.—The presence of penicillinase was determined by noting the presence of growth in tubes containing sterile filtrate plus penicillin and comparing with the control tubes containing the same concentration of penicillin. It was found that, with the method used, the highest concentration of penicillin in which growth of *Staph. aureus*, strain H, was permitted by culture filtrates was 31.25, 12.5 and more than 400 units per ml for strains no. 161R, 196 and 147 respectively. In the control tubes, containing no culture filtrate, growth occurred in tubes containing 0.06 units per ml of penicillin. Penicillinase was not found in filtrates of strains no. 43R, 161 and 43.

The effect of penicillin on toxin production

Little work has been reported on the effect of penicillin on bacterial toxins. Positive results, however, were obtained by Boor and Miller (1945) and Miller and Boor (1946) who were able to protect mice and rabbits against meningococcal endotoxin and mice against gonococcal endotoxin by the repeated administration of large doses of penicillin, and by Mason (1945) who

found that 5,000 to 20,000 units per ml of penicillin could inhibit the coagulase activity of staphylococci.

In the following experiments an attempt was made to determine the effect of penicillin on the growth and toxin production of 6 strains of staphylococci. All strains produced alpha-hemolysin, whereas strain no. 196 was the only one which also produced potent beta-hemolysin. The effect of penicillin on production of staphylococcal toxins has been tested in three mediums, Amigen liquid, Amigen semi-solid agar and veal infusion semi-solid agar.

Preparation of toxin in Amigen liquid medium.—Sterile 100 ml amounts of medium in 500 ml Florence flasks were inoculated with 0.1 or 0.2 ml of a 24 hour culture of the test strain. When penicillin was added it was dissolved in sterile Amigen liquid medium and added aseptically to selected flasks in desired concentrations. Sublethal concentrations of antibiotic, which were different for individual strains, were used in these experiments: strains no. 147, 196, 161R and 43R were grown in 200, 50, 1,000 and 10 units of penicillin per ml respectively. The flasks were closed with sterile one-hole rubber stoppers containing a constricted glass tube through which 20% carbon dioxide gas was added manometrically. The tube was then sealed in a flame and the flasks were rotated on a vertical wheel during incubation for three days at 37 C. Thereafter, the contents of similar flasks were pooled and the bacterial cells were removed by centrifugation. The supernatant fluid, thus collected, was stored in the refrigerator until used in assays for toxin. To determine whether growth was as good in mediums containing penicillin as in mediums without penicillin, viable bacterial counts or cultures grown for three days at 37 C in Amigen liquid medium and in Amigen semi-solid agar, with and without antibiotic, were made. No differences were found in these counts.

Amigen semi-solid agar.—This medium was prepared by adding 0.7% agar to Amigen liquid medium, the p_H adjusted to 7.6 and the medium dispensed in 65 ml amounts into Kolle flasks and sterilized in the autoclave. The antibiotic was added after the agar had cooled to 45 C in order to prevent its destruction by heat. After inoculation the cultures were grown in a desiccator in an atmosphere of 20% carbon dioxide for three days at 37 C. The contents of similar flasks were

TABLE 4.—Effect of penicillin on production of hemolysins by staphylococci.

Medium	Staphylococcus strains	Rabbit Erythrocytes hemolysin (M.H.D./ml)*		Sheep Erythrocytes hemolysin (M.H.D./ml)*	
		Penicillin	No penicillin	Penicillin	No penicillin
Amigen liquid	161		64		
	161R	<2	32-64		
	147	<2	32-64		
	196	<2	32-64	<2	32
	43	†	32		
Amigen semi-solid agar	43R	4	16		
	161		128		
	161R	64	64		
	147	128	128		
	196	128	128	32	128
Veal infusion semi-solid agar	43		64		
	43R	16	8		
	161		64		
	161R	8	64		
	147	128	128		
	196	64	256	64	512
	43		128		
	43R	32	64		

* M.H.D. (minimal hemolytic dose) of hemolysin indicates least amount of toxin which completely lyses 1.0 ml of a 1% saline suspension of erythrocytes after incubation for 1 hour at 37 C (rabbit r.b.c.) followed by overnight refrigeration (sheep r.b.c.).
† Blank spaces in this and subsequent tables indicate no test done.

then shaken into a gauze bag, the agar was broken up and the bag gently squeezed to express as much fluid as possible. This liquid portion collected by centrifugation was used in toxin assays.

Veal infusion semi-solid agar.—This medium was prepared in the same manner as described for Amigen agar except that veal infusion broth was used as the base. The method for preparation of toxin from this medium was identical with that described for Amigen agar.

TABLE 5.—Effect of penicillin on production of lethal factor by staphylococci.

Medium	Staphylococcus strains	Filtrates containing penicillin	Filtrates containing no penicillin
Amigen liquid	161		4/4 (1.0)
	161R	0/4 (1.0)*	4/4 (1.0)
	147	0/4 (1.0)	4/4 (1.0)
	196	0/4 (1.0)	4/4 (1.0)
	43		4/4 (1.0)
Amigen semi-solid agar	43R	0/4 (1.0)	3/4 (1.0)
	161		4/4 (0.5)
	161R	0/4 (1.0)	4/4 (0.5)
	147	4/4 (0.5)	4/4 (0.5)
	196	4/4 (0.5)	4/4 (0.5)
Veal infusion semi-solid agar	43		4/4 (0.5)
	43R	4/4 (1.0)	4/4 (1.0)
	161		4/4 (0.5)
	161R	4/4 (0.5)	4/4 (0.5)
	147	4/4 (0.5)	4/4 (0.5)
	196	4/4 (0.5)	4/4 (0.5)
	43		4/4 (0.5)
	43R	4/4 (0.5)	4/4 (0.5)

* Numerator = number of mice killed within 4 days, denominator = number of mice injected; parenthetic figures = dosage in ml.

Assay of toxins.—The supernatant fluids prepared by the above methods were assayed for selected toxic moieties as follows:

Alpha-hemolysins were assayed in terms of a minimal hemolytic dose (MHD) according to the method of Dolman and Kitching (1935). Beta-hemolysins (hot-cold lysin) was similarly assayed except that the culture fluid was heated at 60 C for one hour prior to testing and sheep erythrocytes were used instead of rabbit cells.

Culture fluids were assayed for lethal factor by intravenous inoculation of 15–20 g white mice with 0.5 to 1.0 ml of toxin. Control mice received similar volumes of uninoculated medium. Although most mice died in less than 4 hours, death within 4 days was considered indicative of lethal toxin.

One-tenth ml amounts of 1 to 10 and 1 to 20 saline dilutions of culture fluid were inoculated intracutaneously into rabbits to test for dermonecrotxin. Control inoculations of phosphate buffer, saline solution, Amigen liquid medium and penicillin were made in each rabbit. The largest area of necrosis and/or redness which developed within 4 days was determined.

Enterotoxin was assayed by monkey feeding test in which 50 ml amounts were fed by stomach tube to monkeys of proven susceptibility, and by the intravenous injection of cats weighing at least 1,000 g, with 2 or 3 ml of culture fluid boiled for 30 minutes. Vomiting during a 5 hour period in the monkeys and a 3 hour period in the cats was considered positive for enterotoxin (Davison, Dack and Cary, 1938; Dack, 1943).

Results.—From tables 4, 5, 6 and 7 it will be seen that the formation of alpha- and beta-hemolysins, lethal factor and dermonecrotxin by staphylococci in Amigen liquid medium was inhibited by penicillin, whereas no such inhibition of enterotoxin formation occurred. However, little or no inhibition of any toxin formation occurred in Amigen or veal infusion semi-solid agar. The failure to obtain more positive results for enterotoxin in monkeys which were fed the toxic supernatants of cultures grown in Amigen liquid and agar mediums may be attributed to the relatively low susceptibility of these animals to enterotoxin and to development of some resistance as a result of previous enterotoxin assays. Since these

results showed inhibition of the formation of toxins other than enterotoxin in cultures grown in Amigen liquid medium containing antibiotic, experiments were devised to determine the neutralizing effect of penicillin on preformed toxin.

Neutralization of preformed toxin by penicillin.—To 10.0 ml amounts of toxin, sterilized by filtering through fritted glass filters, grade UF, penicillin was added to make a final concentration of 1,000 units per ml. Untreated toxin was used as the control. Following preliminary titrations of alpha-hemolysin and lethal toxin the tubes were incubated at 37 C. After one hour, one, two and three days, samples of toxin were removed aseptically and assays were made of the two toxic factors. No loss in titre was detected in either toxin incubated with penicillin or in toxin without penicillin and it was concluded that penicillin had no neutralizing effect on preformed staphylococcal alpha-hemolysin or lethal toxin.

Effect of penicillin on the physiological characteristics of staphylococci

Gallardo (1945) working with penicillin-fast strains of staphylococci found some reduction of coagulase production but no change in the fermentation of

TABLE 6.—*Effect of penicillin on production of dermonecrotxin by staphylococci.*

Medium	Staphylococcus strains	Dermal reaction*	
		Filtrates containing penicillin	Filtrates containing no penicillin
Amigen liquid	161		+
	161R	0	+
	147	±	+
	196	0	+
	43		+
	43R	0	±

* + = area of necrosis and redness 5 mm or more in diameter.

± = area of redness < 5 mm.

0 = no reaction.

mannitol. North and Christie (1945) tested 159 strains of staphylococci and found no correlation between resistance to penicillin and biochemical properties. Gale and Taylor (1946) found that the assimilation of glutamic acid by staphylococci was prevented in cultures grown in the presence of penicillin. Graessle and Frost (1946) in experiments with streptomycin- and penicillin-resistant strains of staphylococci found that some of the streptomycin-fast strains showed a change in pigment production and a reduction in the rate of carbohydrate fermentation, but reported no similar experiments with penicillin-fast strains. Phillips and Barnes (1942) tested staphylococci resistant to gramicidin and found one strain which failed to ferment mannitol and lactose after being made resistant, although the original strain fermented both sugars.

TABLE 7.—*Effect of penicillin on production of enterotoxin by staphylococci.*

Medium	Staphylococcus strains	Monkey feedings (Dosage = 50 ml)		Cat intravenous injections (Dosage = 2-3 ml/kg)	
		Reaction*		Reaction*	
		Penicillin	No penicillin	Penicillin	No penicillin
Amigen liquid	161		0/4		1/2
	161R	0/4	1/3	1/2	1/2
	147	0/5	0/3	1/2	1/2
	196	0/2	1/2	1/2	1/2
Amigen semi-solid agar	161R	0/2	0/2	1/2	1/2
	147	0/2	0/2	2/2	2/2
	196	0/2	0/2	2/2	2/4
Veal infusion semi-solid agar	161R	2/3	2/3		
	147	2/3	—		
	196	1/3	1/3		

* Numerator = number of animals vomiting; denominator = number of animals tested

In the following experiments, the effect of penicillin on the physiological characteristics of selected strains of staphylococci was determined.

The physiological characteristics of the original staphylococcal strains used in these experiments were determined by inoculation of veal infusion broth containing 0.5% dextrose, lactose, sucrose, maltose and mannitol with bromcresol-purple as the indicator. Litmus milk and gelatin were also inoculated. The tubes were incubated at 37 C and readings made after 72 hours. At the same time, 10 of the enterotoxic and 2 of the non-enterotoxic strains were tested for coagulase production.

Results.—All strains formed acid in

penicillin (1,000 units per ml) than in the same mediums without antibiotic.

The experiment was repeated with similar results. To investigate further these results, comparisons were made of the amount of growth and total acid produced in cultures grown in medium containing lactose and glucose.

Two groups of flasks of Amigen liquid medium containing glucose (0.25%) or lactose (0.5%) with and without penicillin (1,000 units/ml) were prepared. Duplicate flasks were incubated in the presence and absence of 20% of carbon dioxide. All flasks were inoculated with 0.1 ml of a 24 hour culture of strain no. 161R in Amigen liquid medium. Uninoculated flasks with and

TABLE 8.—*p_H* of cultures of staphylococci grown in presence and absence of penicillin.

Sugar	Strain*							
	161	161R		147		43	43R	
		Penicillin	No Penicillin	Penicillin	No Penicillin		Penicillin	No Penicillin
Dextrose	4.30	4.54	4.19	4.40	4.34	4.36	4.38	4.27
Mannitol	5.58	5.81	5.22	5.57	5.25	5.11	4.54	5.24
Lactose	5.92	6.42	5.42	4.71	4.49	3.95	4.76	4.76
Sucrose	4.49	5.36	4.40	5.07	4.70	4.45	5.01	4.83
Maltose	4.81	5.44	4.68	5.02	4.80	4.47	4.48	4.76

* Strains 161R, 147 and 43R grown in presence of 1,000, 200 and 5 units per ml of penicillin respectively.

the sugar broths, liquified gelatin and the 12 which were tested produced coagulase.

Characteristics of strains during contact with penicillin—Comparisons were made of the reactions in sugar broths of strains no. 161, 161R, 147, 43 and 43R when grown in the presence and absence of penicillin. After 72 hours incubation at 37 C, *p_H* readings of the cultures were taken with a Cameron *p_H* meter. Duplicate tubes of each sugar medium were inoculated with the culture under test. The results of these tests are summarized in table 8. The figures represent an arithmetic average of the *p_H* readings obtained from duplicate cultures. The maximum variation was 0.25 *p_H* unit. It will be noted that a higher *p_H* was present in cultures of strain no. 161R grown in lactose, sucrose and maltose broths containing

without penicillin were also prepared. Control counts of the inoculum and initial *p_H* readings were made. Samples from each flask were removed aseptically after 24, 48, 72 and 96 hours, one week and two weeks (96 hour determinations were not made from lactose cultures) for viable counts, *p_H* readings and acid titrations. In those flasks incubated with carbon dioxide, the gas was renewed after each sampling. Viable counts were made by the pour plate method using veal infusion agar. Acid titrations with N/10 sodium hydroxide with brom-thymol-blue as an indicator were made on 3 ml samples of culture from which the cells had been removed by centrifugation and the carbon dioxide driven off by boiling.

Results.—Figures 2, 3, 4 and 5 summarize in graphic form the results obtained with cultures of strain no. 161R grown in glucose and lactose Amigen liquid mediums. Experiments with glucose and lactose mediums were done as two separate tests at different times and the inoculum in each test was different. The control inoculum into the glucose

medium was 4.8 (log of number of bacteria per ml) and was, thus, too low to indicate in figures 2 and 3.

It will be seen that the viable counts of organisms grown in the two mediums were comparable regardless of the presence of antibiotic. Although no appreciable difference in p_H or acidity was present in cultures grown in mediums containing glucose, with or without penicillin, a definite difference is seen in

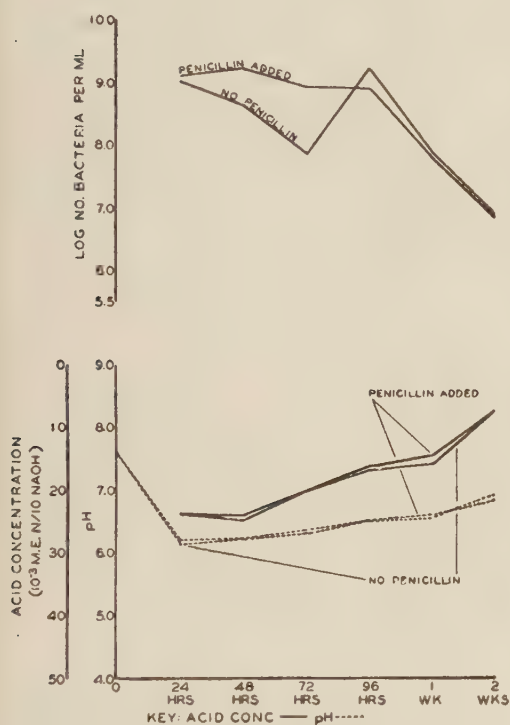


FIG. 2.—Effect of penicillin on fermentation of glucose (strain 161R grown with 20% carbon dioxide).

cultures grown in mediums containing lactose. It is evident that the presence of penicillin resulted in less acid production in cultures grown in the lactose medium (figures 4 and 5). These results suggest that penicillin interferes with the fermentation of the disaccharide, lactose, and probably with the fermentation of other disaccharides.

Since lactose is composed of galactose

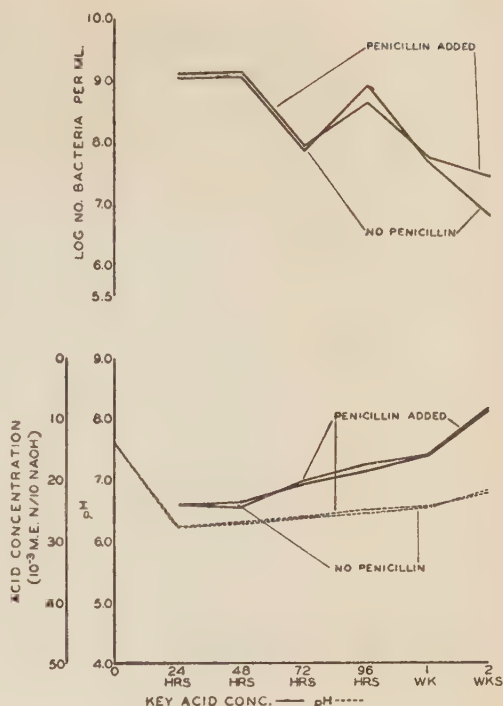


FIG. 3.—Effect of penicillin on fermentation of glucose (strain 161R grown without carbon dioxide).

and glucose, experiments were done to determine the p_H of cultures of strain no. 161R grown in Amigen liquid medium with and without penicillin containing 0.25 and 0.5% galactose as compared to similar cultures grown in the same concentration of glucose. The results obtained with these experiments indicate that, although the organisms grew well in all of the mediums, the cultures fermented 0.5% galactose much more slowly than 0.5% glucose. In the presence of penicillin the fermentation of galactose was further inhibited. No such inhibition of glucose fermentation occurred in the presence of penicillin. Further experiments were not done with sucrose and maltose and no explanation is offered for the reduction in acid formation of cultures grown in these sugars in the presence of sublethal concentrations of penicillin.

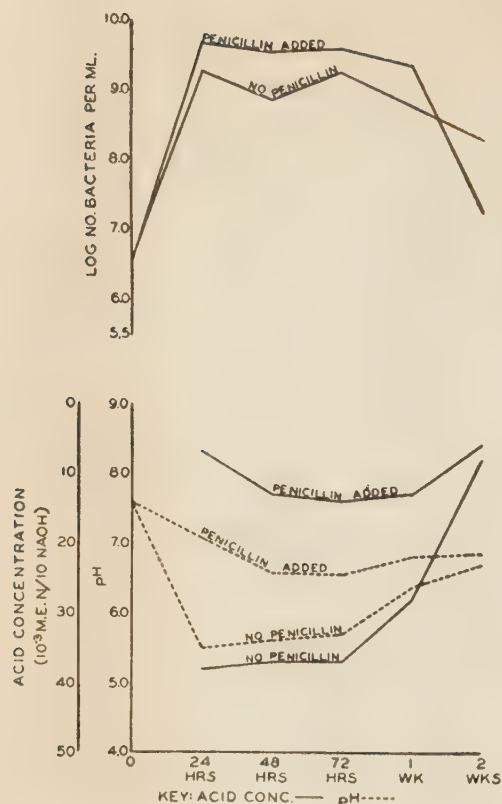


FIG. 4.—Effect of penicillin on fermentation of lactose (strain 161R grown with 20% carbon dioxide).

The effect of penicillin on coagulase activity.—Since few reports have appeared on the effect of penicillin on coagulase activity of staphylococci (Mason, 1945; Gallardo, 1945) experiments were done to test such activity in the strains selected in these studies.

Strains no. 161R, 147 and 196 were grown in Amigen liquid medium with and without penicillin in the manner previously described. After incubation for three days at 37 C the cultures were centrifuged and the supernatant toxin decanted. The cells were resuspended in saline solution, washed three times in sterile saline and finally resuspended to a turbidity of a 24 hour culture. This final suspension was used as one source of coagulase. The decanted toxic material from the above cultures was divided into two portions and one part was filtered through a Berkefeld N filter. The resulting filtrate and the

unfiltered toxin were also used as sources of coagulase. The coagulase test was performed by adding 0.125 ml of the material to be assayed to 0.5 ml of fresh rabbit plasma diluted 1 to 4.

Results.—The above experiments showed the presence of coagulase in all of the substances assayed. Differences were noted only in the length of time required for coagulation to occur, the maximum time being 17 hours. Delay

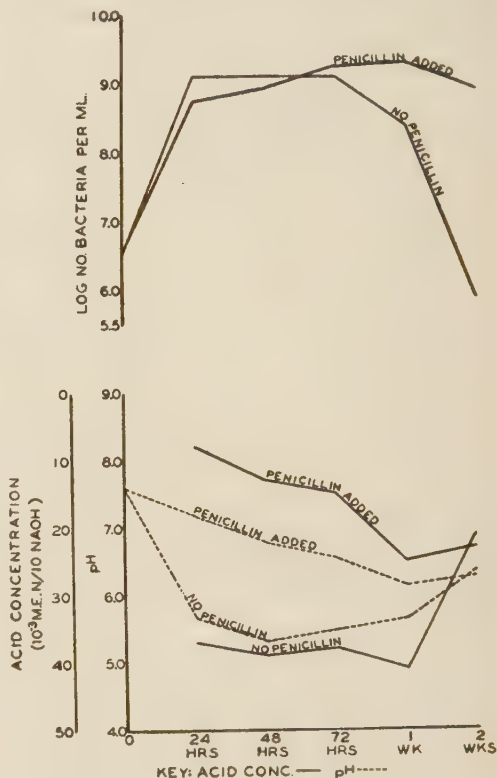


FIG. 5.—Effect of penicillin on fermentation of lactose (strain 161R grown without carbon dioxide).

was observed only in the case of the unfiltered toxin of strain no. 147 and the Berkefeld filtrate of no. 196 from cultures grown in the presence of penicillin.

DISCUSSION

Tests for sensitivity of staphylococci to penicillin have been done mainly on strains isolated from infectious processes

before, during and after penicillin therapy, on normal stock strains and on strains used for assay of the antibiotic. Few authors have reported assays of sensitivity on food poisoning staphylococci, and the finding of so high a proportion of resistance to penicillin among the fifteen enterotoxic strains reported in the present study makes it necessary that tests be carried out on many more food poisoning staphylococci. The paucity of experimental data relating to sensitivity of enterotoxic staphylococci to the antibiotic makes it impossible to draw any general conclusions regarding other food poisoning staphylococci and their resistance to penicillin. For the present, it can only be said that this resistance is not evident in all enterotoxic strains since cultures sensitive to concentrations as low as 0.05 units per ml were found. Since many strains of staphylococci were shown to be resistant to the action of penicillin, the use of the antibiotic in the control of food poisoning caused by staphylococci would be impractical.

The development of increased resistance in staphylococci to penicillin both in vivo and in vitro has been demonstrated repeatedly (Demerec, 1945; Gallardo, 1945; McKee and Houck, 1943; North and Christie, 1945; Rake, et al., 1944; Rammelkamp and Maxon, 1942; Spink and Ferris, 1945; Spink, Ferris and Vivino, 1944; Todd, Turner and Drew, 1945.) These same reports, however, have brought forth results which, in some instances, have been inconsistent. In 1940, Abraham and Chain reported that a substance inhibiting penicillin, which they called penicillinase, could be obtained from extracts of *E. coli* and by the same method of extraction they could get no such substance from staphylococci. Thereafter, Florey et al. (1941) made staphylococci artificially resistant to penicillin and,

using the extraction method of Abraham and Chain (1940), were also unable to demonstrate the enzyme. In addition, they found that staphylococci were still inhibited when grown in the fluid of *E. coli* cultures containing penicillin, indicating that no destruction of penicillin occurred. Bondi and Dietz (1944) tested different bacteria and found that many produced penicillinase but were unable to detect such a substance from 5 strains of staphylococci. These authors made no mention of the degree of natural susceptibility of these 5 strains and, hence, may have tested very sensitive organisms. These same authors (1944a) developed artificial resistance in one strain of staphylococcus, *E. typhi* and *Proteus* and found no production of penicillinase. They concluded that the development of resistance to penicillin was not due to the ability of a strain to produce the enzyme. Kirby (1944) examined 7 naturally resistant and 7 susceptible strains of staphylococci and by alcohol and ether extraction obtained penicillinase from all of the resistant strains and no enzyme from any of the sensitive ones. Spink and Ferris (1945) found that 4 strains of staphylococci, made resistant in vivo, produced penicillinase while 4 other strains, made resistant in vitro, produced no penicillinase. These same authors also reported that the in vitro resistant strains regained their sensitivity on repeated subculture in penicillin-free mediums, while the in vivo resistant strains did not. Todd et al. (1945) and Graessle and Frost (1946) also developed increased resistance in staphylococci to penicillin in vitro and reported the same return to sensitivity upon repeated subculture in penicillin-free mediums. Woodruff and Foster (1945) isolated species of *Bacillus* which produced potent penicillinase and found that just as much extra-cellu-

lar enzyme was produced in the presence of penicillin as in its absence and concluded that it must be a constitutive enzyme. Gots (1945) examined a number of naturally resistant strains of staphylococci including some strains isolated from patients undergoing penicillin therapy and found penicillinase in all cultures resistant to more than one unit of penicillin per ml.

In the present study, penicillinase was found in two naturally resistant (147 and 196) and one artificially resistant (161R) strains of staphylococci but not in another artificially resistant strain (43R) or in the susceptible parent cultures (161 and 43). Insofar as the author is aware this is the first report indicating the presence of penicillinase in a strain of staphylococcus made resistant to penicillin *in vitro*. It should be pointed out that the strain used in this study was far more resistant to penicillin than were those used by others in the study of penicillinase production in artificially resistant strains. From the experimental results obtained with strain 161R on the determinations of the proportion of cells resistant to varying concentrations of penicillin it is evident that the strain is not homogeneous in its capacity for resistance. The counts indicate that only a small proportion of the cells are resistant.

In another type of experiment, Demerec (1945) developed *in vitro* resistance to penicillin in one strain of staphylococcus and from the results of his experimental data summarized "... that resistance is not induced by the action of penicillin on bacteria but originates through mutation and that penicillin acts as a selective agent to eliminate non-resistant individuals." Furthermore, this same author claims that strains, isolated after passage in penicillin-containing mediums, acquired a permanent resistance, a fact not cor-

roborated by others (Todd, Turner and Drew, 1945; Spink and Ferris, 1945).

The present results obtained with strain 43R confirm, in part, the results of Demerec (1945). If one examines the data in table 3, it will be seen that a very high proportion of cells are resistant to penicillin and yet penicillinase was not detected in this strain by the methods used. This is an entirely different result from that obtained with the experiments on strain 161R. It will be recalled that penicillinase was produced by the latter strain and plate counts indicated that only a small proportion of the individual cells were resistant to the antibiotic. It thus appears that resistance to penicillin may be due to one or several factors acting singly or in combination. Two of these factors have been demonstrated in these experiments, namely, the resistance of staphylococci due to their ability to produce penicillinase and resistance presumably due to selection of resistant cells. It is apparent that variations in susceptibility among strains of the same species may be due to differences in ability to produce penicillinase. It is also apparent that resistant strains may be developed through selection by using penicillin to eliminate non-resistant cells. But, selection of cells able to produce penicillinase may also be a factor, as has been demonstrated here with an *in vitro* artificially resistant strain and by others in *in vivo* developed resistant strains.

Reports on the effect of penicillin on bacterial toxins have been few. While attention in the present experiments was particularly directed towards learning the effects of penicillin on enterotoxin, the antibiotic effects on the other toxins of staphylococci were noteworthy as the experimental results demonstrated. Since enterotoxin formation proved unaffected by penicillin in all

of the three mediums tested, this may be considered as additional evidence which distinguishes enterotoxin from the other staphylococcal toxins. However, the fact that inhibition of formation of the other toxins tested occurred in Amigen liquid and very little, if at all, in Amigen and veal infusion semi-solid agar poses an intriguing problem. It has long been known that the addition of agar to a culture medium resulted in better toxin production by staphylococci (Burnet, 1930, 1931; Bigger, 1933; Leonard and Holm, 1935; McClean, 1937; McIlwain, 1938). Thus, it is interesting to note that in the experiments reported here, the addition of agar to Amigen liquid apparently prevented penicillin from exerting the effect produced in the agar-free mediums. The fact that inhibition of toxin formation by penicillin occurred in Amigen liquid cultures is impossible to explain at this time. Much further work is required before any conclusions can be reached.

The occasional reports which have appeared attempting to correlate penicillin resistance and biochemical properties in staphylococci have been negative (North and Christie, 1945; Gallardo, 1945). However, two studies have been reported which indicate some effect of penicillin on the metabolism of staphylococci. Gale and Taylor (1946) showed that penicillin prevented the assimilation of glutamic acid in staphylococci growing in penicillin medium and Welshimer, Krampitz and Werkman (1947) reported that penicillin interferes with the dismutation of pyruvate although this action was later attributed to impurities in the penicillin preparations (Krampitz and Werkman, 1947). The present experiments also report no differences in biochemical properties of staphylococci when comparisons were made of strains before and after being

made resistant to penicillin. However, it was found that when cultures of an artificially resistant strain (161R) were grown in lactose, sucrose or maltose broths to which penicillin had been added in sublethal concentrations, less acid was formed than when penicillin was absent. No such differences were found in cultures grown in glucose medium with and without antibiotic. Comparisons of growth of the same strain in lactose and glucose Amigen liquid medium revealed comparable viable counts while the p_H of the cultures growing in lactose was affected by the presence of penicillin (figs. 4, 5, 6 and 7). From these experiments it seemed that the fermentation of lactose was inhibited by the antibiotic. Further experiments with the component monosaccharides of lactose, galactose and glucose, indicated that galactose fermentation produced little acid in cultures containing either 0.25% or 0.5% concentration of the sugar. Experimental results obtained with 0.25% glucose also indicated a low acid production. At the present time, no mechanism of action is offered which would explain the inhibition of acid formation of cultures grown in galactose and lactose mediums containing penicillin nor for the same type of inhibition occurring in sucrose and maltose mediums.

SUMMARY

The natural resistance of 15 enterotoxigenic and 5 non-enterotoxigenic strains of staphylococci to penicillin was determined. The enterotoxigenic strains were found to have a wide range of resistance to penicillin (0.05 to 500 units per ml) while the non-enterotoxigenic strains were found to be relatively sensitive (0.05 to 0.1 unit per ml) to the antibiotic. Artificial resistance was developed in one enterotoxigenic and one non-enterotoxigenic strain to 2,000 and 10 units of penicillin per ml respectively. Under the experi-

mental conditions stated, production of penicillinase was demonstrated in two naturally resistant and one artificially resistant strains, but not in one artificially resistant nor in both parent strains of the two artificially resistant cultures. In the artificially resistant enterotoxigenic strain, resistance was associated with the production of penicillinase, whereas the resistance of the non-enterotoxigenic strain was apparently due to selection of resistant cells without penicillinase formation.

Rabbit and sheep red blood cell hemolysin, lethal factor and dermonecrotxin production of both naturally and artificially resistant strains of staphylococci was inhibited when the organisms were grown in Amigen liquid medium containing sublethal concentrations of penicillin. No such inhibition of toxin formation by penicillin occurred in Amigen or veal infusion semi-solid agar. Enterotoxin production was not inhibited in any of the three mediums by the addition of penicillin.

It was found that the addition of penicillin to preformed staphylococcal toxin did not result in neutralization of the toxin.

The effect of penicillin on the biochemical activities of naturally and artificially resistant strains of staphylococci was determined. Interference with the fermentation of the disaccharides, lactose, sucrose and maltose in actively growing cultures was demonstrated.

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